Saltwater based fractionation and valorisation of macroalgae

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SHORT TITLE: Saltwater fractionation of macroalgae

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jctb.6443

ABSTRACT

BACKGROUND:

Macroalgae is gaining increasing interest as an important biomass feedstock. Yet when valorising marine biomass, the presence of salt can pose a substantial obstacle to the effectiveness of downstream biological and chemical processes, as well as the engineering infrastructure required. Accordingly, dewatering, washing and drying is often considered the first and crucial primary step in processing marine biomasses such as microalgae and macroalgae. These high costs can make further marine biorefinery commercialisation prohibitive. This investigation assesses simple pre-treatments for macroalgal biomass in saltwater, thereby reducing the fresh water footprint and removing the need for an energy intensive washing and drying stage.

RESULTS:

Using acid and basic catalysts, the carbohydrate and soluble protein components were fractionated into a soluble aqueous phase, for further fermentation and a solid phase suitable for hydrothermal liquefaction. The presence of saltwater was found to aid the fractionation process, solubilising more of the biomass. The use of H_2SO_4 produced more monosaccharides, while NaOH solubilised higher levels of biomass at lower temperatures. The aqueous phase was demonstrated to be suitable for biological processing with the salt tolerant yeast *M. pulcherrima*, and the residual solids suitable for processing via hydrothermal liquefaction (HTL).

CONCLUSION:

In contrast to existing pre-treatment strategies, we demonstrate an entirely salt-based biochemical conversion route is a potentially viable option. For the first time this work demonstrates that, rather than a hindrance, the presence of saltwater can be advantageous, and could provide an alternative, more cost effective pathway to achieve a successful macroalgal based biorefinery.

NOMENCLATURE:

DCW: Dry cell weight

FS: *Fucus serratus*

HTL: Hydrothermal liquefaction

$m_{starting\ FS}$: Initial starting mass of FS

$m_{oven\ dry\ pellet}$: Mass of oven dried biomass pellet

MP: *Metschnikowia pulcherrima*

OD_{600}: Optical density of light absorbed at 600 nm wavelength

PLS: Partial least squares regression

TGA: Thermogravimetric analysis

TN: Total nitrogen

TOC: Total organic carbon

MG: Mannose, and galactose combined sugars

INTRODUCTION

Global population growth coupled with a shift towards a low-carbon economy means that biomass resources are of increasing importance. Macroalgae (seaweed) have been identified as a sustainable biomass resource for the production of food, biofuels, and biochemical building-blocks for the chemicals industry.[1] With higher photosynthetic efficiencies than terrestrial crops, and no freshwater or arable land requirement, macroalgae is an attractive alternative to terrestrial biomass. While mass cultivation is still a relatively immature technology in Europe (~1,500 tonnes per year),[2] the potential is huge with over 30M tonnes currently cultivated globally.[3] Wild harvests have remained steady in recent decades, at approximately 1M tonnes per annum globally.[3] In contrast to cultivation, European activity accounts for almost a third of this global wild harvest volume. Species with attractive biochemical and/or physiochemical properties have been identified as potential sources of chemicals, particularly in pharmaceuticals where marine polysaccharides such as carrageenan and fucoidan are compounds of interest.[4]

For lower value compounds, macroalgal biomass has been shown to be a suitable source for hydrothermal liquefaction, fermentations and oleaginous yeast production.[5] However, to exploit macroalgal biomass for lower bulk chemical and fuel production, a number of issues must still be addressed across every part of the supply chain. One of the key issues with the valorisation of macroalgae, which is rarely discussed, is the presence of salt. The presence of salt can pose a substantial obstacle with regards to the effectiveness of downstream biological and chemical processes, as well as with the

engineering infrastructure required. Accordingly, expensive dewatering, washing and drying is often considered the first and crucial primary step in processing marine biomasses such as microalgae and macroalgae. These high costs can make further marine biorefinery commercialisation prohibitive. As such, marine biomass research in the most part is carried out with fresh-water washed samples.[6] Similarly, post-harvest freshwater washing of macroalgae is deemed vital for hydrothermal processing as salt water heated to effective processing temperatures is extremely corrosive and will degrade any steel type rapidly. If processing biologically, most industrially relevant microbes identified for the production of monomers and fuels are not salt tolerant and cannot grow under such conditions.[7]

Hydrothermal liquefaction (HTL) has shown excellent promise as a conversion technique for the production of fuels, and aqueous fertilisers.[8] However, treatment processes like HTL are indiscriminate in their hydrolysis of carbohydrate structures, resulting in the complete loss of high-value polysaccharides. Similarly, fermentations are typically only able to use up to 50 % of the organic fraction depending on the saccharide composition and yield.[5]

Fractionation of biomass into individual building block components allows extraction of the high-value products, as well as optimal treatment of the different value fractions for maximum value recovery. Work in the lignocellulosic biorefinery field has demonstrated that the pre-treatment and fractionation of the biomass is of critical importance to direct mass flow between processing steps, to optimally valorise the biomass, and to employ the most suitable downstream process for the fraction.[9] The distribution of fermentable material into a readily assessable aqueous phase is a

key priority, while left over the solids can be processed thermochemically. Such a method has additional benefits: the presence of common heteroatoms (e.g. nitrogen and sulphur) in the whole biomass can lead to undesirable NO_x and SO_x emissions when combusting the biofuel products, as well as difficulties in processing and handling of biocrudes.[10,11] However, the presence of nitrogen or sulphur containing compounds in the aqueous or solid residues produced by thermochemical processing may be desired, with both the aqueous phase and solids being identified as potential fertilizer products for soil amelioration.[10,12] Previously, biomass pre-treatment techniques to reduce nitrogen content in the resulting bio-crude from HTL have been successfully demonstrated, however at the expense of requiring an additional aqueous processing step.[10]

Fractionation techniques for the separation of lignin, cellulose, and hemicellulose, are well demonstrated in the literature for lignocellulosic biomass, though the fractionation of macroalgal species less so. Macroalgae is typically comprised of cellulose, polysaccharide carbohydrates, and a small quantity of lipids and proteins,[13] so should be amenable to similar processing techniques to those applied to lignocellulosic biomass. Typical treatments applied to macroalgae include dilute acid or base treatment, mixed aqueous/organic solvent washings, or use of chelating salt solutions to either dissolve carbohydrates in solution or to enhance the properties of the residual solids fraction by selective stripping of components, e.g. agar or alginate.[14–19] The presence of salt in raw seaweed biomass, however, provides uncertainty as to the effectiveness and efficacy of processing and the only previous work in this area has evaluated the effect of both residual marine salts and inorganic

catalytic salts on the HTL process, rather than investigating the role of salt water in the fractionation process.[20–23] Literature reports typically tends to favour dilute acid treatment as the preferred chemical treatment of algal biomass to produce hydrolysates.[22,24–26] Primarily, this is due to the desirable mechanism of acid hydrolysis on structural polysaccharides: cleavage of the linkage between individual monosaccharide units under acidic conditions results in a high sugar concentration.[17] However, typical acid hydrolysis conditions are harsh (1–10% acid w/v, 120–200 °C), and could be problematic for the design of industrial-scale reactors, a key concern being the risk of material corrosion.[17] Acid hydrolysis is also associated with the risk of producing toxic inhibitors, for example the degradation of hexose sugars, i.e. glucose, producing 5-(hydroxymethyl)furfural (5-HMF).[27] However, some studies have reported lower production of inhibitory side-products when treating lignocellulosic biomass with inorganic salts during hydrolysis/saccharification pre-treatments.[28]

Microbial fermentation is a platform unit operation in both conceptual biorefineries, and many existing industrial biorefinery processes. Typically, a combination of pre-treatment and hydrolysis techniques are employed to produce a broth fermentable sugars.[29] A multi-step process is often required to ensure complete the hydrolysis of poly- and oligo-saccharides, since many industrially suitable microbes are not able to fully metabolise the various complex oligosaccharides resulting from one-step hydrolysis reactions of untreated-biomass.[30–33] Additionally, detoxification steps are often required to remove or neutralise inhibitory compounds that are produced as co-products of the hydrolysis reactions.[34,35] However, recent work has demonstrated the yeast *Metschnikowia pulcherrima* is able to metabolise both

complex water soluble oligosaccharides, and mono-/di-saccharides sourced from varied biomass sources (agricultural, aquatic and industrial), as well as exhibiting high tolerance to inhibitors typically found in lignocellulosic hydrolysates. [36–38] Additionally, *M. pulcherrima* is found to produce a number of desirable products, such as 2-phenylethanol, and high-value lipids.[37] Previous studies have shown that optimal *M. pulcherrima* cultures have elevated levels of NaCl (up to 6 g L^{-1}), however the salt tolerance of *M. pulcherrima* at marine salinities (circa. 35 g L^{-1} NaCl) has not been reported on.[39]

Given the gap in literature relating to salt water fractionation, this study aims to evaluate simple macroalgal fractionation reactions in model seawater and assess the impact of salt on typical downstream processes, in this instance, HTL of residual solids, and fermentation of the aqueous hydrolysate.

MATERIALS AND METHODS

Model Seawater

A single 10 L batch of seawater was prepared for use in all experiments by dissolving Aquarium Systems Reef Crystals salt in deionised (DI) water (~40 g L^{-1}). Temperature-corrected specific gravity was checked and adjusted to 1.025 (equivalent to 35 ppt seawater) using a D&D H_2Ocean brand handheld salinity refractometer. A single batch of brine (70 ppt) was made up by dissolving salt in DI water at approx. 80 g L^{-1}. Brackish water (10 ppt salt) was made up by mixing at an approximate 3:7 volume

ratio of seawater (35 ppt) and DI water (0 ppt) and confirming with specific gravity measurement of 1.007 to 1.008.

Macroalgae

Samples of the macroalgae *Fucus serratus* (*FS*) were wild-harvested from Broadsands Beach, Paignton (50°24'24.9"N, 3°33'16.2"W) between November 2017 and January 2018. Samples were frozen at -80°C and then freeze dried at -55°C (Coolsafe, Scanvac) and then triturated to a fine powder, sieved to < 1.4mm, and stored in sealed containers at -80°C to prevent degradation.

Acid/base catalyst solutions

An 11M NaOH stock solution was prepared by dissolving Fisher BioReagent brand ≥98% NaOH pellets in DI water at 452 g L^{-1}. A 1.8M sulfuric acid stock solution was prepared by making 5.0 mL conc. H_2SO_4 (99.99%) (Sigma Aldrich brand) up to 50 mL with DI water in a volumetric flask. For the acid/base concentration screening experiment (Fig. 1a and 1b), a double strength concentrate was made up to maintain the same total reaction volume.

10 mL scale hydrolysis reaction

ACE pressure tubes (15 mL) were used as reaction vessels, with constant agitation provided by a 10mm x 5mm crossbar magnetic stirrer. Biomass loading rates of *FS* 5 wt.% were used to ensure optimal stirring.[10]

Temperature control was achieved by placing an insulated aluminium block heat sink on a stirrer hotplate, with machined recesses to snuggly accept the 15mL ACE pressure tubes (25.4mm O.D.). The aluminium block was pre-heated to the target reaction temperature, allowing a period of no less than 20 min for the block temperature to stabilise once the thermal set-point was achieved. Temperature control was achieved by way of an IKA ETS-D5 electronic contact thermometer inserted into a drilled thermowell in the centre of the reaction block.

Solvent of varying salinity (freshwater, brackish water, seawater, or brine) was mixed with aqueous catalyst solution (NaOH or H_2SO_4) at a volume ratio of 10:2 to give a total of 12 mL working volume in the pressure tube, to which 0.51 g of *FS* biomass was added, along with a 10mm x 5mm crossbar magnetic stirrer. The pressure tube was then quickly sealed, placed into the pre-heated reaction block on the stirrer-hotplate, and continuously stirred by the magnetic crossbar for 20 min. Once the reaction time had elapsed, the pressure tubes were removed from the heated reaction block and placed into a room-temperature aluminium block to rapidly quench the temperature. The contents of the reaction tube were transferred to a pre-tared 50mL Falcon centrifuge tube and adjusted to pH 5–6 using HCl/NaOH.

An initial catalyst strength screening experiment was performed. Stock solutions of NaOH and H_2SO_4 were prepared, using the literature convention of wt% for NaOH and a v/v% for H_2SO_4 to allow direct comparison across the literature.[10] Such that a 2mL addition of NaOH solution to 10 mL of water resulted in 0.5, 1.0, 2.8, 6.8, 11.0, and 13.6 wt. % active NaOH in the final 12 mL reaction volume. Similarly, stock solutions of H_2SO_4 were prepared such that 2 mL volume addition of the stock solution

resulted in 0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 v/v % H_2SO_4 in the total reaction mixture. Catalyst strength screening was carried out at 87.5 °C, in both fresh (0 ppt) and salt (35 ppt) water.

Influence of reaction temperature, salinity, and choice of catalyst on the hydrolysis yield was investigated by use of a multi-factorial screening study. Experimental design was conducted in the software package Modde Pro (version 12.1), with continuous independent factors of reaction temperature (25 to 150 °C), and salinity (0 ppt to 70 ppt, fresh water to heavy brine), and categorical factors of choice of catalyst (acid, base, none), catalyst strength was kept constant. A full summary of reaction solvent volumes, catalyst concentrations, and temperatures is provided in the Supporting Information (Table S1).

Yield calculation

The conversion yields of biomass solids were determined for the hydrolysis reaction. The reaction solution was centrifuged, and the supernatant aspirated into a 30 mL glass sample vial. The resultant solid was resuspended in 6 mL DI water with the use of a benchtop vortex mixer and centrifuged again. Supernatant from the washing was transferred to the 30 mL glass vial along with the primary aqueous fraction. The centrifuge tube with the wet pellets of solids was then placed in a laboratory oven (Plus II Oven, Gallenkamp) at 60 °C until constant weight was achieved.
Conversion yield (%) was calculated as follows:

Equation 1

$$Yield_{conversion} = \frac{\left(m_{starting\ FS} - m_{oven\ dry\ pellet}\right)}{m_{starting\ FS}} \times 100\%$$

Where m is the mass (g) of starting *FS*, and the oven dried pellet. Ash-free conversion yield (%) was later calculated using the ash content (%) determined by TGA as follows:

Equation 2

$$Yield_{Ash-free} = \frac{\left(m_{starting\ FS} - \left[m_{oven\ dry\ pellet} \times (100\% - ash\%)\right]\right)}{m_{starting\ FS}} \times 100\%$$

Where $ash\%$ is the ash content of solids as determined by TGA, full method for which is explained later.

Media preparation for fermentation

A pre-inoculum broth was prepared by inoculating 10 mL of soy-malt broth (SMB: soy peptone 30 g L^{-1}; malt extract 25 g L^{-1}; pH 5; in DI water) with a single colony of *Metschnikowia pulcherrima* NCYC 4331 (National Collection of Yeast Cultures, Norfolk, UK), from an agar plate (YMD: yeast extract 10 g L^{-1}, malt extract 20 g L^{-1}, glucose 20 g L^{-1}, agar 15 g L^{-1}, pH 5 in DI water). The inoculum was prepared in a 100mL unbaffled Erlenmeyer (shake) flask, incubated for 24 h at 20°C in temperature-controlled cabinets, at a 10% (v/v) working volume, and agitated on orbital shakers (Unimax 2010, Heidolph) at 180 rpm.[5]

96-well plate fermentation

Fermentability was assessed using 96-well plates. Plates (Greiner BioOne Cellstar, suspension culture plate, F-Bottom with lid) were prepared as follows: 7.5 µL of tetracycline/gentamicin stock solution (960 mg L^{-1} tetracycline; 1200 mg L^{-1} gentamicin) was combined with 285 µL of macroalgae hydrolysate, and inoculated with 7.5 µL (2.5 % v/v) inoculum (giving a final working volume of 300 µL per well, containing 12 mg L^{-1} tetracycline and 15 mg L^{-1} gentamicin). Plates were loosely covered with a plate lid, incubated at 25 °C at 200 rpm idle continuous shaking frequency (BMG Labtech, CLARIOstar Plus) for 132 hours (5.5 days), with light absorbance at 600 nm wavelength (OD_{600}) analysed every 30 minutes, following 300 s of 200 rpm double orbital shaking to ensure sample homogeneity. The initial reading of OD_{600} for each well was subtracted from the subsequent and final OD_{600} readings.

5 L scale hydrolysis reaction

In a stirred and jacketed reactor with a 5 L working volume (Parr Instrument Company), seawater (574.9 mL), freshwater (1804.0 mL), and catalyst solution (276.5 mL 11M NaOH) were added to make a total reaction volume of 2655 mL brackish water at 4.3 wt. % NaOH. This mixture was brought to reaction temperature (90 °C) under vigorous stirring (500 rpm stirrer speed), and temperature maintained by PID control. On reaching reaction temperature, the solid macroalgae was added (450 g, equivalent to ~13.5 wt. %), and the reaction stopwatch started. Samples (approx. 100 mL per) were extracted from the vessel using a ½" sampling ball-valve fitted at the base of the reactor, collected at 5-minute intervals starting at t=20 min until t=90 min.

Full summarised details of the reaction charge are detailed in the supporting information (Table S2).

5 L scale hydrolysate clarification and work-up

Samples drawn from the Parr 5 L reactor were transferred to 250 mL wide-mouth HDPE centrifuge bottles (Nalgene), and centrifuged for 30 minutes (RCF = 2990). Supernatant was transferred to fresh tared centrifuge tubes (50 mL conical base, Falcon), and pH adjusted to <5.0 using 10M and 1M HCl to minimise the total volume of HCl added. Primary solids in the 250 mL centrifuge bottles were set aside for processing later.

The pH <5.0 adjustment of the primary supernatant caused significant precipitation of solids; hence, solutions were centrifuged for a further 30 min (RCF = 2990). Secondary supernatant was again transferred to a fresh tared centrifuge tube, and pH adjusted to >10.1 using powdered $Ca(OH)_2$ (calcium hydroxide, 98%, extra pure, ACROS Organics™). The addition of $Ca(OH)_2$ caused further precipitation; the secondary supernatant was returned to the centrifuge for a further 30 min (RCF = 2990) to isolate the secondary solids. Secondary solids were retained.

The tertiary supernatant was a clear straw-coloured liquid, and did not require any further clarification. The pH was adjusted to pH 5 with HCl, the clarified pH 5 hydrolysate was transferred to 100 mL shake flasks (Erlenmeyer flask), and autoclaved at 121 °C in preparation for inoculation with *M. pulcherrima* inoculum solution.

Shake flask cultivation

Autoclaved hydrolysate was prepared in 100 mL shake flasks as described in the previous section, and a solution of inoculum was prepared as described previously under "Media preparation for fermentation": 1 mL of hydrolysate was taken and set aside for use as a blank for the OD_{600} measurement. The *M. pulcherrima* inoculum was prepared such that the OD_{600} of the fermentation broth would be equal to 0.5 immediately after inoculation. To determine the required volume of inoculum for each shake flask the following equation was used:

Equation 3

$$V_{inoculum\ required} = \frac{0.5 \times V_{hydrolysate\ in\ flask}}{OD_{600_{inoculum}}}$$

Where V is volume (mL), and OD_{600} is the optical density of the sample at 600 nm wavelength of light.

OD_{600} of the inoculum was assessed against a blank of DI water, and the volume of hydrolysate in the shake flask assessed from the level gradients on the side. Inoculated shake-flasks were incubated at 20 °C in temperature-controlled cabinets, and agitated on orbital shakers (Unimax 2010, Heidolph) at 180 rpm. 1 mL samples of the fermentation broth were extracted daily for 6 days (144 hrs), and analysed for OD_{600} against the blank of pre-inoculated hydrolysate previously collected. Between measurements, the blank was frozen, and when defrosted was homogenized on a bench-top vortex mixer before measurement.

Determination of dry cell weight (DCW) was completed as described by Abeln et al.[5] Samples of the culture were centrifuged, supernatant aspirated using a micropipette and set aside, pellet re-suspended in DI water, centrifuged again, and the wash-water supernatant discarded. DI water washes were repeated until the supernatant was clear of colour. The subsequent water-wet pellet was frozen (-20 °C), lyophilised, and dry-weight gravimetrically assessed.

Hydrothermal liquefaction (HTL)

Primary solids from 5 L scale fermentation work-up were resuspended in equal volume of DI water, neutralised to pH 5 to 7 with 1M HCl, and centrifuged for 10 minutes. Supernatant washings were discarded, and the washed solids were dried in a laboratory oven (Plus II Oven, Gallenkamp) at 60 °C until constant weight.

Hydrothermal liquefaction (HTL) was performed on the solids as described by Raikova et al.[8] Batch reactors previously fabricated in the group using stainless steel tubing with Swagelok® fittings were used. The tubular reactor was loaded with 1.5 g of oven-dried primary solids and 13.5 mL of DI water, and heated within a vertical tubular furnace until the reaction temperature of 350 °C was reached, then removed from the furnace and allowed to cool to room temperature.

After cooling, gaseous products were released via the needle valve into an inverted, water-filled measuring cylinder to measure gaseous fraction volume. Gas phase yields are calculated using the ideal gas law, and approximating the gas phase as 100 % CO_2, with a molecular weight 44 g mol^{-1}, and ideal volume 22.465 dm^3 mol^{-1}, as

previously demonstrated by Raikova et al.[8,11,40] The yield of gaseous product was determined with the following:

Equation 4

$$yield_{gas} = \frac{\left(V_{gas} \times 1.789 \times 10^{-3}\right)}{m_{dry\ biomass}} \times 100\%$$

Following this, the aqueous phase was decanted from the reactor contents and filtered through a Fisher qualitative filter paper pre-dried overnight at 60 °C. The product yield in the water phase was determined by leaving a 2.5 g aliquot of the aqueous phase to dry in a 60 °C oven overnight and scaling the residue yield to the total aqueous phase mass. Aqueous phase residue yield was determined using the following: [8]

Equation 5

$$yield_{aqueous\ residue} = \frac{m_{residue}}{m_{dry\ biomass}} \times 100\%$$

The remaining biocrude and char phase was washed from the reactor using chloroform until the solvent ran clear, and filtered through the same filter paper used to separate the aqueous phase (after drying with an air stream to ensure evaporation of residual water). The filter paper and collected char were washed thoroughly with chloroform to remove all remaining biocrude. The filtrate was collected, and solvent removed under vacuum (40 °C, 72 mBar) until no further solvent evaporation was

observed visually. Biocrude samples were transferred to 30 mL vials using a small volume of chloroform; solvent was removed *in vacuo*, and vials were left to vent to atmosphere *via* a needle for a further 12 h to remove residual solvent. Biocrude yield was determined using the following equation: [8]

Equation 6

$$yield_{biocrude} = \frac{m_{biocrude}}{m_{dry\ biomass}} \times 100\%$$

The solids char yield was calculated from the mass of the retentate collected on the filter paper after drying overnight in an oven at 60 °C. Solid yield was determined as follows: [8]

Equation 7

$$yield_{solids} = \frac{m_{solid}}{m_{dry\ biomass}} \times 100\%$$

Characterisation

Thermogravametric analysis (TGA) was performed on a Setaram Setsys Evolution TGA 16/18. The Calisto programme was used to collect and process data. Samples were individually loaded into a 170 µL alumina crucible. Under an argon atmosphere, samples were held at room temperature for 20 min, with temperature ramped to 800 °C over 20 mins, and held at 800 °C under argon for 30 min. The atmosphere was then

swapped to air whilst maintaining a temperature of 800 °C for 40 min, followed by cooling to ambient temperature over 20 min. Ash content was determined by taking the final mass at the end of the 40 min under air, and expressing as % of the starting mass.

Equation 8

$$Ash\ \% = \frac{m_{800°C\ residue}}{m_{starting\ starting}} \times 100\%$$

LC-MS analysis was performed using a Dionex UltiMate 3000 LC system (Thermo Scientific). Liquid chromatography method was performed was as published by Sluiter et al.[41] A BioRad Aminex HPX-87H column was used, with an injection volume 50µL, a mobile phase of 5mM sulfuric acid flowing at 0.6 mL min^{-1}, and column temperature set to 60 °C. Detection was made by quadrupole time-of-flight (QTOF) MS, using a Bruker MaXis HD II spectrometer, with the ESI in negative ion mode.

Samples were prepared for LC by centrifuging in 1.5 mL Eppendorf tubes at 13,500 rpm for 10 min, then passing through a 0.22 µm PTFE membrane syringe filter. The clarified hydrolysate was then diluted 1:10 with 0.22 µm filtered DI water.

Elemental analysis (CHN) of solids was carried out externally by OEA Laboratories Limited (OEA Laboratories Limited, Unit C2, Florence Road Business Park, Kelly Bray, Callington, Cornwall, PL17 8EX, United Kingdom). Elemental analysis was carried out in duplicate for each sample, and the average value reported.

Total organic carbon (TOC) and total nitrogen (TN) analysis of aqueous samples were carried out with an automated TOC-L analyser (Shimadzu) fitted with a TNM-L

total nitrogen analyser unit and an ASI-L autosampler. Analysis was carried out in triplicate and average values reported.

Optical density (OD) of the fermentation broth was assessed at 600 nm (OD_{600}) in a spectrophotometer (Spectronic 200, ThermoFisher Scientific), using 4.5 mL disposable polystyrene cuvettes (Fisherbrand™), against a blank of pre-inoculated hydrolysate collected before inoculation and frozen in between measurements (unless otherwise stated). Samples of fermentation broth were diluted with DI water to an OD_{600} in the range 0 to 1, and the final OD_{600} reported was equal to:

Equation 9

$$OD_{600_{reported}} = OD_{600_{instrument}} \times [dilution\ factor]$$

Samples that had been frozen prior to measurement were defrosted quickly in a water bath, and vortexed on a benchtop vortex mixer to homogenise before measurement.

RESULTS AND DISCUSSION

Effect of hydrolysis reaction conditions

Hydrolysis of *F. serratus* biomass was carried out at 87.5 °C, under acidic and basic conditions, in either fresh- or saltwater. The conversion of biomass to soluble products is presented in Fig. 1.

For both acid and base catalysts, conversion yield tends to increase with catalyst concentration, although a plateau is observed above 7.0 wt.% NaOH for both fresh- and saltwater conditions (Fig. 1b), beyond which no improvement in conversion is seen. Hydrolysis performance in saltwater is similar to that in freshwater for acid catalysis, but saltwater was found to enhance the efficiency of conversion for the NaOH catalyst substantially relative to freshwater.

Fig. 1 confirms that the catalyst strengths used by Hu et al. of 2.0 v/v % H_2SO_4 and 7.0 wt. % NaOH in their microalgae study, were appropriate for this study in macroalgae.[10] Catalyst loadings of 2.0 v/v % H_2SO_4 and 7.0 wt. % NaOH were therefore selected for the multi-factorial screening study of temperature, salinity, and catalyst choice. Ash-free yields are summarised in Fig. 2.

The average ash-free conversion yields ranged between 43.0 % and 93.9 %, depending on the conditions selected. Temperature was observed to have a strong effect on the overall ash-free hydrolysis yield, with higher temperature resulting in higher conversion yields. At low and mid salinity values, base-catalysed conditions tended to give the best conversions, although uncatalysed became more effective at extremely high salinity (heavy brine solutions with salt concentrations of 70 ppt).

Carbon, hydrogen, and nitrogen (CHN) composition of the solid residues, and total organic carbon (TOC) / total nitrogen (TN) of the aqueous hydrolysates were individually carried out (Tables S3 and S4 in supplementary information). Carbon content of the residual solids ranged between 37.32 % and 54.85 %, hydrogen content ranged between 5.11 % and 8.96 %, and nitrogen ranged between 0.91 % and 2.79 %. Comparisons between variables were made with a two-sample unequal variance *t*-test.

Temperature was observed to have a strong influence on carbon content of the residual solids, with higher temperature resulting in higher carbon content for all catalysts and salinities (an average of 40.32 % at low temp vs. 46.73 % at high temp, *p*-value 0.004), however no similar global temperature correlation was observed for hydrogen or nitrogen content. Catalyst choice showed no significant influence on carbon content in the solids, however, base treatment at high temperature produced solids with a higher hydrogen content than acid treatment (average of 5.45 % under high-temp acid treatment vs. 7.97 % under high-temp base treatment, *p*-value 0.040), and base treatment in both low and high temperature resulted in lower nitrogen content in the solids compared to both acid and uncatalysed hydrolysis reactions (average low temperature acid nitrogen content 1.93 % vs. low temperature base treatment 1.48%, *p*-value 0.018, and high-temperature acid nitrogen content of 2.28 % vs high-temperature base nitrogen content 0.94 % *p*-value 0.008 for low and high temperatures respectively).

Total organic carbon (TOC) and total nitrogen (TN) concentrations in the aqueous hydrolysate followed a similar pattern, with TOC ranging between 2,875 mg L^{-1} and 11,063 mg L^{-1}, and TN ranging between 136 mg L^{-1} and 609 mg L^{-1}. Higher temperature reactions consistently resulted in a higher concentrations of both TOC and TN compared to the lower temperature for all catalyst and salinities. Catalyst choice, unlike the carbon content of residual solids, had a large influence on the TOC in the hydrolysate, with base treatment resulting in higher TOC in the hydrolysate for all temperatures and salinities. Again, the influence of base treatment on the nitrogen was significant, with base treatment resulting in higher TN in the hydrolysate

compared to both acid and uncatalysed treatments, and all temperatures and salinities.

Carbon/nitrogen ratio was used as a proxy for the effect of experimental conditions on the carbohydrate/protein fractionation. Higher carbon/nitrogen ratio in a particular phase suggests a relative concentration of carbonaceous compounds (e.g. carbohydrate residues) compared to nitrogen, conversely a lower carbon/nitrogen ratio in a phase suggests a relative higher concentration of nitrogenated compounds (e.g. protein residues). High C/N ratio is desired in the solids, as this indicates a lower concentration of heteroatoms (e.g. N, S, O) and a likely better quality HTL bio-crude product. Conversely a low TOC/TN ratio is desired in the hydrolysate, since this indicates a higher concentration of nitrogenated compounds in the aq. phase, which could be beneficial for the use of the aqueous phase as a fertiliser.

Main effects plots of C/N ratio in the solids, and TOC/TN in the hydrolysate were produced in Minitab v 18.1 (Fig. S1, supplementary info) which show that for high C/N in the solids, and low TOC/TN in the aq. phase, base treatment has the strongest single effect, followed by high temperature. Focusing just on main effects however can miss some important interaction details, for example if the presence of salt influenced the action of the base treatment. To investigate the interaction effects of temperature, salinity, and catalyst choice on the relative carbon and nitrogen partition in the residual solids and hydrolysate, main effects plots and interaction plots were produced in Minitab v 18.1 (Figure 3).

Fig. 3a shows the interaction plots of the three experimental variables on the C/N wt % ratio in the residual solids. Parallel lines on an interaction plot suggest no

interaction between the plotted variables, however diverging, converging, or crossing lines indicates that there's an interaction between the two plotted factors. The interaction of catalyst choice and salinity (Fig. 3a,i) shows that the highest C/N ratios achieved at lower salinities, and lower C/N ratios at higher salinity. The relatively parallel lines in the interaction plot for salinity and temperature (Fig, 3a,ii) show that there is not any particularly strong interaction between these two factors, however the increased spacing at high temperature does suggest that any salinity specific effects (higher C/N ratio at low salinities) are enhanced at higher temperature. The interaction effect of temperature and catalysts (Fig. 3a,iii) showed again a strong interaction, with the combination of high temperature and base having the strongest effect on C/N ratio enhancement, however, little to no effect for the acid catalysed hydrolysis, and a slight decrease in the C/N ratio with increasing temperature for uncatalysed reactions. Fig. 3b shows the interaction plots of the three experimental variables on the TOC/TN ratio in the aqueous hydrolysate. The gapping between points on the cross interaction plot of salinity and catalyst choice (Fig. 3b,i) shows that the specific impact of salinity on the TOC/TN (from Fig. S1b, lower salinity tends to result in higher TOC/TN, higher salinity tends to result in lower TOC/TN) is less pronounced under base catalysed conditions. The cross interaction plot of catalyst choice and temperature (Fig 3b,iii) on the TOC/TN ratio shows that under acid and base treated reactions the effect of temperature is to decrease the TOC/TN ratio in the hydrolysate, however, under uncatalysed conditions the effect of higher temperature is to increase TOC/TN in the hydrolysate – in the absence of a catalyst to assist in protein

depolymerisation, increasing temperatures enhance the dissolution of easily accessible carbohydrates, effectively concentrating the nitrogen in the solid phase.

A multi-variate partial least squares (PLS) model of temperature vs. salinity vs. catalyst was subsequently developed in Modde Pro (version 12.1), using ash-free conversion yield, aqueous TOC and TN, residue solids C % and N % (and the corresponding aqueous TOC/TN and solid C/N ratios) as response factors. 4-dimensional response contour plots of ash-free yield vs. temperature, salinity, and catalyst type are presented in figure 4, and a summary of fit plot with further model detail is available in the supplementary information (figure S2). In all cases, increasing temperatures resulted in greater biomass conversion to aqueous phase products; yields were also enhanced by the presence of salt in solution. This model supports the findings of the interaction plots: base-catalysed processing is most sensitive to temperature variation, whereas acid-catalysed and catalyst-free processing is sensitive to both temperature and salinity. Similar 4D response plots were produced for TOC/TN ratio in the aqueous phase and C/N ratio in solids phase (see supporting information, Fig. S3). These also suggest that reactions under basic conditions result in the highest C/N ratio in the solids (resulting in low-nitrogen solids, beneficial for use of the solid as an HTL feedstock), and lowest TOC/TN ratio in the aqueous phase (which could be beneficial for use of the aqueous phase as a fertiliser).

Analysis of soluble fraction

The soluble fraction can potentially be used as a fermentation media, as such the saccharide composition of the hydrolysate was assessed by LC-MS (fig. 5). Mannitol,

fucose, glucose, rhamnose, mannose, galactose, fructose, maltose, and xylose were quantified, based on the fucoidan carbohydrate structure of *Fucus serratus* described by Bilan et al. and the microwave hydrolysis of brown seaweeds by Abeln et al.[5,42] Xylose, mannose, and galactose co-eluted on the column used, however use of the MS detection allowed separation of the pentose (xylose) and hexose (mannose, galactose), Mannose and galactose are presented as a combined, single MG fraction, and fructose/maltose/xylose were summed together as mixed sugars on Fig. 5, as their concentration was very low. Oligosaccharide concentration was quantified in comparison to a cellobiose standard for ease of comparison across all experimental conditions.

The various hydrolysis conditions show similar effect at liberating mannitol from the biomass structure, with mannitol found in all samples at concentrations ranging from 1.8 g L^{-1} to 3.5 g L^{-1}. Presence of salt was not seen to have a major impact on the concentration of mannitol recovered, with an average mannitol concentration of 2.7 g L^{-1} and 2.4 g L^{-1} for 0 ppt and 35 ppt salinities respectively. Similarly, the influence of temperature showed a relatively small difference, with lower temperature hydrolysis yielding slightly more mannitol than high temperature (2.7 g L^{-1} and 2.3 g L^{-1} for 25 °C and 150 °C respectively). Choice of catalyst showed an interesting trend however, with the choice of no catalyst yielding the highest average mannitol concentration (3.0 g L^{-1}), compared to acid (2.5 g L^{-1}), and base (2.0 g L^{-1}) hydrolysis conditions. The decrease in mannitol concentration at elevated temperatures and under catalysed conditions (acid, base, and/or salt) suggest that mannitol released into solution is undergoing further reaction to inhibitory products. Yamaguchi reports that mannitol dehydration

to various degradation products progresses uncatalyzed in water at high temperature (573 K), or under sulfuric acid conditions at 377 K.[43] Despite the clear indication of degradation of the easily soluble saccharides under acid/base conditions, the total saccharide yield for non-catalysed hydrolysis was typically much lower than either acid or base for all temperatures and salinities.

The quantity of oligosaccharides in solution was found to be higher in the samples hydrolysed in saltwater conditions, and the concentration of monosaccharides (fucose, glucose, MG, and mixed sugars) was unsurprisingly highest for acid-treated samples, due to elevated hydrolysis of the more resistant biomass structures.

The mixed broth of mono-, di-, and oligosaccharides, with variable salt content, would be unsuitable for the fermentation of a common yeast such as *Saccharomyces cerevisiae*. However, previous research has shown the capability of the highly tolerant yeast *Metschnikowia pulcherrima* to catabolise a wide range of C5 and C6 monosaccharides and macroalgal oligosaccharides, as well as being extremely tolerant to the presence of inhibitors and elevated salt.[5,37,39,44] To screen for the suitability of the hydrolysates for growing *M. pulcherrima*, the yeast was cultured on the hydrolysates, with no additional nutrients.

Fermentation screen of *Metschnikowia pulcherrima* using *Fucus serratus* hydrolysates

Fermentability was screened using 96-well plates, at a 300 µL working volume scale (Fig. 6). Absorbance at λ = 600 nm (OD_{600}) was recorded to approximate cell density. Measurements taken over the 5.5 day period demonstrated that *M.*

pulcherrima was able to successfully metabolise *F. serratus* hydrolysate produced using the full range of reaction conditions investigated. Final OD_{600} measurements are variable; however, a rapid onset of the exponential growth phase was observed for all conditions, demonstrated by a steep linear rise of OD_{600}. In all cases the lag-phase of the growth curve of the yeast was extremely brief, typically <2 hours, before the onset of the exponential growth phase.

The presence of high quantities of salt in the fermentation broth appeared to have had no impact on the final OD_{600} in all cases. Comparing the performance of *M. pulcherrima* in hydrolysates produced in freshwater (0ppt), saltwater (35ppt), and brine (70 ppt) (Fig. 6g) demonstrates that the effect of even a substantial salt content in the fermentation broth on *M. pulcherrima* growth is negligible. This suggests that saltwater processing of biomass will not negatively affect a downstream fermentation process based around a *M. pulcherrima* platform. Additionally, there is evidence that the presence of salt in the fermentation broth may assist with recovery of target bio-based molecules, such as 1,3-propanediol, 2,3-butanediol, acetoin, and lactic acid.[45]

Acid pre-treatment is known to carry risk of producing toxic degradation products of both hexoses and pentoses.[27,34,46] Nevertheless, growth of *M. pulcherrima* was unaffected in this case, due to its high inhibitor tolerance (Figs. 6a and 6e). Favourably, base processing does not pose the same risk of producing toxic dehydration products from monomer sugars. However, due to the different mechanism of biomass digestion, although overall biomass conversion is higher, the yield of monosaccharides under NaOH treatment is significantly lower than with H_2SO_4. This was confirmed by LC-MS analysis of the hydrolysate, with individual sugars

detected and quantified as described by Sluiter et al. (2011).[41] Despite this, no significant difference in the performance of *M. pulcherrima* in H_2SO_4-treated vs. NaOH-treated biomass hydrolysates was observed across all temperature and salinity conditions (Fig. 6i).

5 L scale-up

From the small-scale screening study, it was concluded that base-assisted hydrolysis at high temperature was optimal, and from the 96-well fermentation screen it is concluded that the washing of harvested biomass to remove salt was not necessary to preserve the growth of *M. pulcherrima*. Fractionation was therefore repeated at a larger lab scale, with the initial hydrolysis performed in a 5 L Parr stainless steel jacketed stirred reactor. Scaling up presented an opportunity to produce a sufficient quantity of residual solids to evaluate the efficacy of performing HTL reactions on residual solids, as well as explore the conversion achieved at times greater than 20 minutes. In order to retain sufficient residual solids after hydrolysis, a conversion of approximately 50 to 55 % during the first 20-minutes was targeted. A salinity of approximately 8.5 ppt was selected in order to model a large scale system using brackish water at an estuary site, and using the 4D response contour plots generated previously (Fig. 4), a reaction temperature of 90 °C was selected. In addition, mechanical agitation afforded an opportunity to increase solids loading, thus solid loading was increased to approximately 13.5 wt. %.

Throughout the reaction, samples were drawn every 5 minutes after an initial 20-minute period. Overall hydrolysis conversion yields ranged between 64 % and 77 %,

with a gradual increase in conversion observed over the 20 to 90-minute reaction time (Fig. 7a). Samples were neutralised using HCl, and solids and aqueous phases separated by centrifugation. The solids were washed with DI water, and processed using HTL. The corresponding aqueous hydrolysate fractions were clarified and then then used as fermentation media for *M. pulcherrima* in 100 mL Erlenmeyer (shake) flasks.

Hydrothermal liquefaction of solid residues

Solid extracts from the 5 L Parr reactor samples were processed using HTL to evaluate their suitability as a feedstock for bio-crude production. HTL reactions were performed as described previously.[8] Mass balances (the yields of bio-crude, solid char, aqueous phase organics and bio-gas) are presented in Fig. 7b. For solids isolated at all stages of hydrolysis, higher bio-crude yields were obtained compared to performing HTL on unprocessed *F. serratus* biomass. Additionally, the yield of solid bio-char was substantially lower for the first Parr reactor sample (25 min) relative to unprocessed *F. serratus* biomass, although this increased with increasing hydrolysis time. As conversion of biomass increases with increasing hydrolysis time, as polysaccharides and oligosaccharides were solubilised, the solid residue became richer in insoluble material with a high ash content, leading to corresponding increases in the yield of HTL bio-char. The exception to this observation being the 80 min sample (Fig. 7b), which shows a decrease in the yield of bio-char and increase in the yield of bio-gas compared to the previous sample (65 min). Comparing the conversion yields for samples at 65

min and 80 min (Fig. 7a), the 80 min sample sits slightly below the general observed trend with a conversion yield of 70.8 %, compared to 73.0 % at 65 min.

The overall mass closure ranged between 78.1 % and 83.0 % (Fig. 7b). The approximate 20 % loss of material is due in part to light organics lost during the work-up of the bio-crude and thermal drying of the aqueous phase, as well as potentially oxygen removal as water into the aqueous phase.[8,47] Overall these mass closures are similar to those observed by Raikova et al. in hydrothermal processing of the macroalgae *A. nodosum*,[8] and by Anastasakis and Ross in hydrothermal processing of *L. hyperborea, A. esculenta, L. digitate, and L. saccharina.*[47]

Shake flask cultivation of M. pulcherrima

Cultivation of *M. pulcherrima* using the NaOH-treated hydrolysates produced at a 5 L scale was carried out on at 100 mL scale in shake flasks. Optical density (OD_{600}) was used to track growth of *M. pulcherrima* over 144 hours total fermentation time (see supporting information Fig. S4, for full kinetic plots). The hydrolysates were fermented with no additional nutrients and the final dry cell weight was not strongly correlated with hydrolysis time, suggesting that a short fractionation reaction could be suitable for rapid NaOH-assisted solvation of the fermentable carbohydrates from macroalgae (Fig. 7c).

Initial growth rates in the 0 to 2 day time frame show a clear difference between hydrolysis times, with fermentation on shorter hydrolysis-time hydrolysates accumulating dry-cell mass significantly faster than longer hydrolysis-time samples. However, by the sixth day all differences in initial grow rates were normalised, with

total accumulated dry-cell weights ranging between 1.4 g L^{-1} and 3 g L^{-1} for all hydrolysis times, similar to previously reported fermentation on microwave assisted hydrolysis of *F. serratus* macroalgae.[5] The pattern of differing growth rates and lag phases is presumably due to lower inhibitors being produced at the shorter reaction times, though the overall level of fermentable carbohydrate in the hydrolysis samples is approximately the same irrespective of hydrolysis time.

CONCLUSIONS

In conclusion, we have demonstrated that it is possible to perform both HTL and fermentation on the products of a saltwater and NaOH treated macroalgae. NaOH as a hydrolysis catalyst offers advantages over dilute acid treatment in two ways: firstly, it selectively strips nitrogen from residue solids leaving a higher quality material for the HTL process; secondly, it presents a much simpler materials compatibility selection problem. The presence of salts in seawater when used as the reaction solvent enhanced the solids conversion yield of the hydrolysis reaction and had no demonstrable effect on the yeast *M. pulcherrima* when the salt-containing hydrolysate solution was used as a fermentation feedstock. Temperature was found to be the single highest influence on biomass solvation yields; however, the prospect of processing in seawater at lower temperatures offers some potential energy savings compared to freshwater processes. Residue solids from brackish and base treatment were advanced to HTL processing, where it was observed that NaOH pre-treatment of

the biomass improved the biocrude yield, as well as decreasing the insoluble biochar product from the HTL reaction. This work provides a crucial missing step towards a true salt based Marine Biorefinery (rather than a biorefinery simply based on marine biomass) by establishing the initial fractionation step, and crucially allowing the direct flow of biomass to HTL/Fermentation products by simply adjusting the temperature of the fractionation reaction.

ACKNOWLEDGEMENTS

This work was partially supported by the Engineering and Physical Sciences Research Council (EP/L016354/1), and though the Industrial Biotechnology Catalyst (Innovate UK, BBSRC, EPSRC) to support the translation, development and commercialisation of innovative Industrial Biotechnology processes (EP/N013522/1). The authors would also like to thank Rosie and Archie Allen for all their expert help in macroalgal harvesting.

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FIGURES

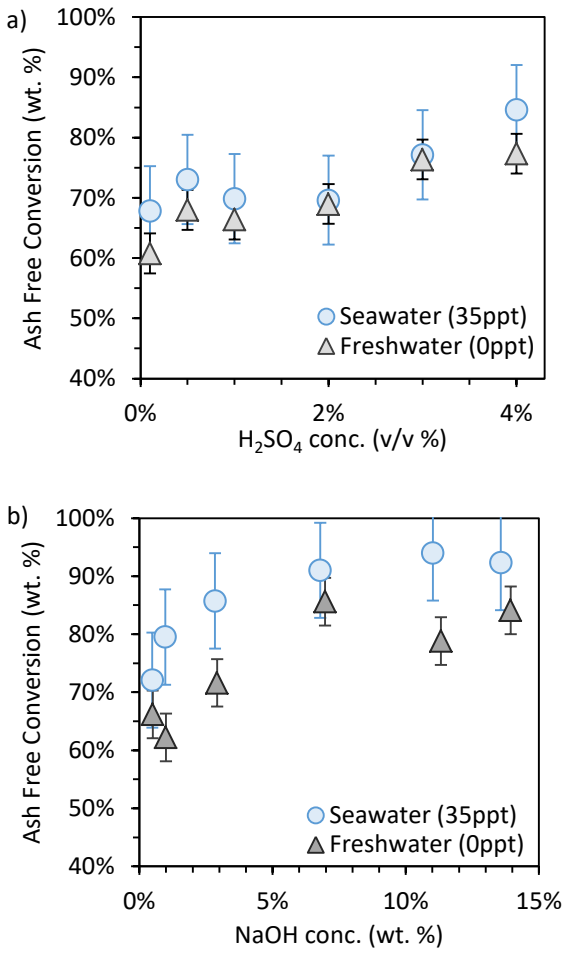

Figure 1: Conversion yields for *Fucus serratus* processed in freshwater (0ppt salt) and saltwater (35ppt), at a reaction temperature of 87.5 °C, total reaction time of 20 minutes, and using (a) a sulphuric acid catalyst; (b) a sodium hydroxide catalyst. Error bars show one standard deviation determined with repeats in the later multi-factorial screening data-set.

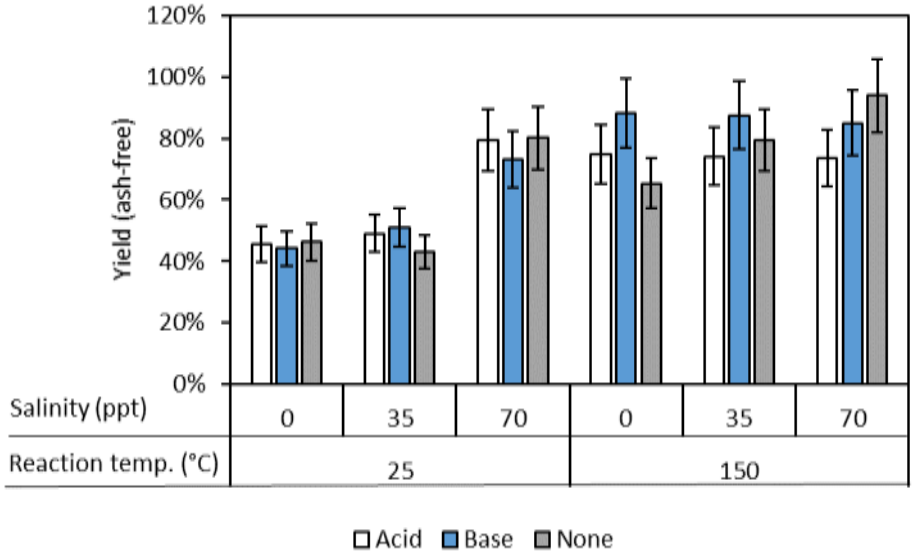

Figure 2: Effect of temperature, salinity and catalyst type on conversion yields from hydrolysis of *Fucus serratus*. Hydrolysis experiments carried out under acid (2 v/v % H_2SO_4), base (7.0 wt. % NaOH), or uncatalyzed conditions, at temperatures 25 °C and 150 °C, and salinities of 0 ppt, 35 ppt, and 70 ppt. All reactions carried out for 20 minutes. Error bars set at the standard error observed in this experiment. Colour available on-line.

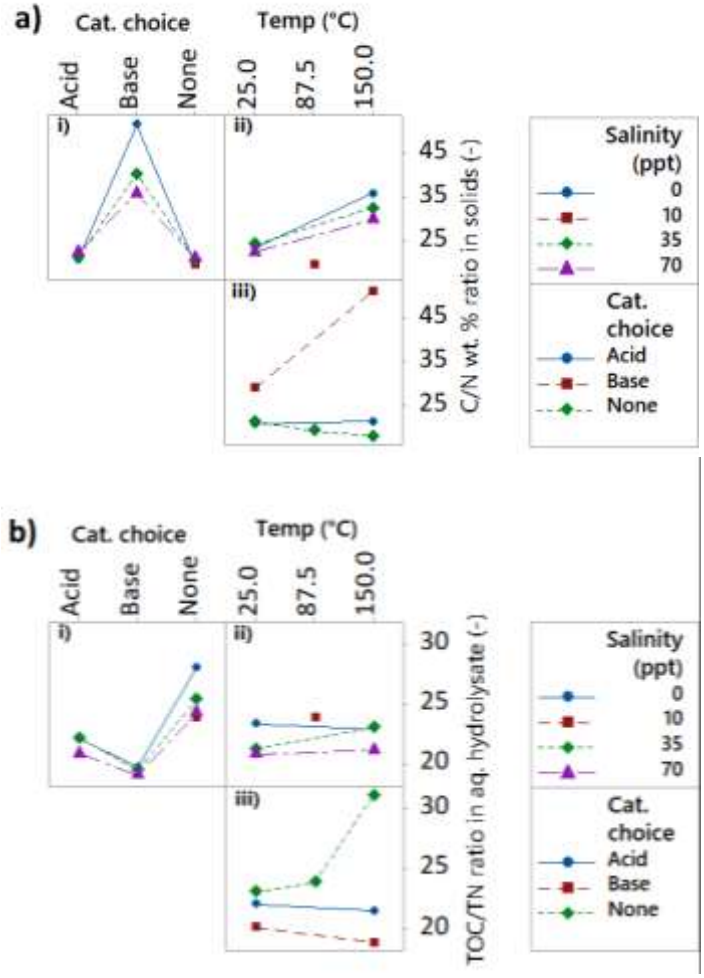

Figure 3: Interaction plots (produced in Minitab v. 18.1) for the effect of salinity, catalyst (cat.), and temperature on, **a)** the carbon/nitrogen wt. % (C/N) mass ratio in the residual solids following fractionation. Subplots **a,i)** showing the relative interaction between catalyst choice and salinity (legend on right), **a,ii)** showing the relative interaction between temperature and salinity, and **a,iii)** showing the interaction between temperature and catalyst choice. For all sub-plots, axis on the right shows the average C/N wt. % ratio measured in the solid residues. **b)** The total organic carbon/ total nitrogen mg L^{-1} (TOC/TN) ratio in the aq. hydrolysate following fractionation. Subplots b**,i)** showing the relative interaction between catalyst choice and salinity (legend on right), b**,ii)** showing the relative interaction between temperature and salinity, and b**,iii)** showing the interaction between temperature and catalyst choice. For all sub-plots, axis on the right shows the average TOC/TN ratio measured in the aq. hydrolysate. Colour available on-line.

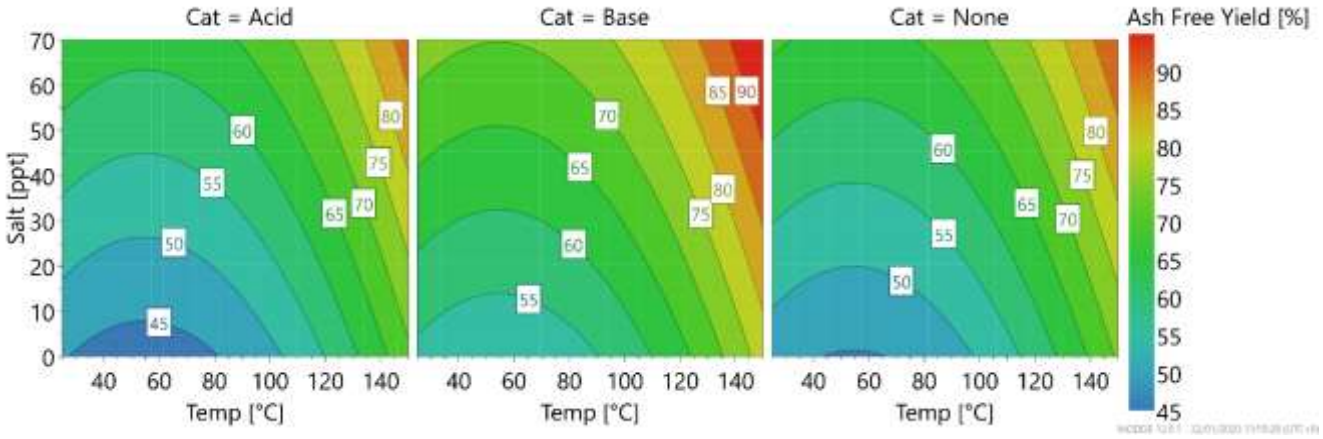

Figure 4: 4-Dimensional response contour plot for multi-factorial model assessing the effect of salinity (0 to 70 ppt), temperature (25 °C to 150 °C), and catalyst choice (Acid, Base, or None) on the predicted ash-free hydrolysis yield (wt. %). Model produced by partial least squares (PLS) regression correlation in Modde Pro v12.0.1, summary of fit plot and further model details is included in the supporting information Fig. S2. Colour available on-line.

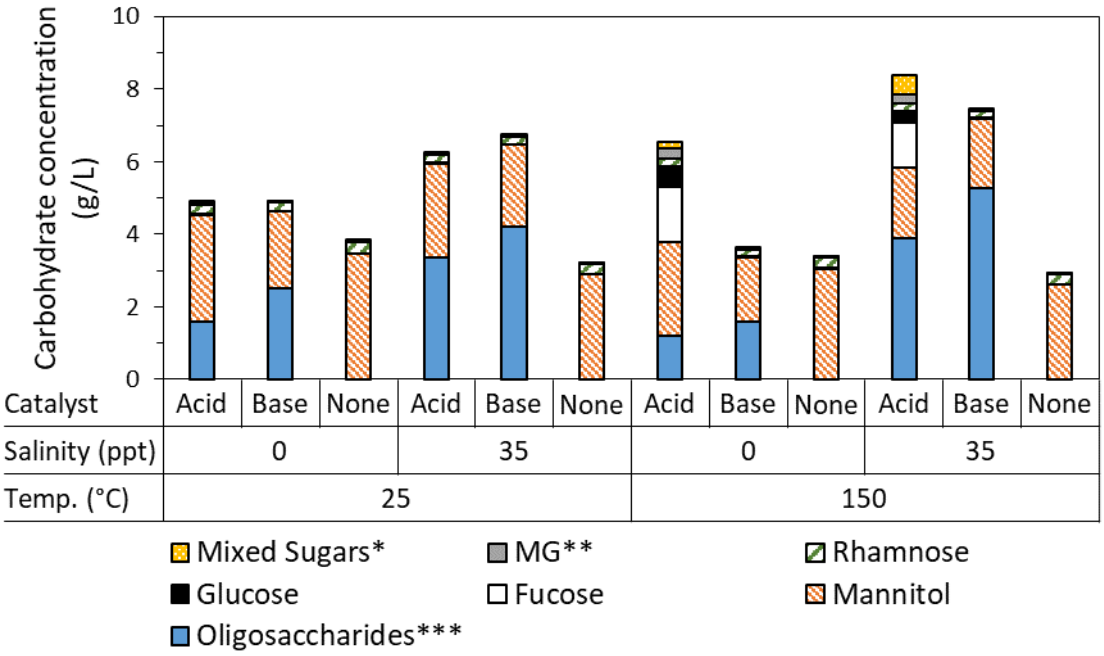

Figure 5: Concentrations of sugars in the hydrolysate determined by LC-MS for different experimental conditions. *Mixed Sugars refers to combined fructose, maltose, xylose saccharide fractions summed together. **MG refers to combined mannose and galactose fractions that co-eluted together and thus were indistinguishable with the techniques used. ****Fucus serratus* oligosaccharide was quantified in comparison to a cellobiose standard for ease of comparison across experimental conditions, thus is an approximation.

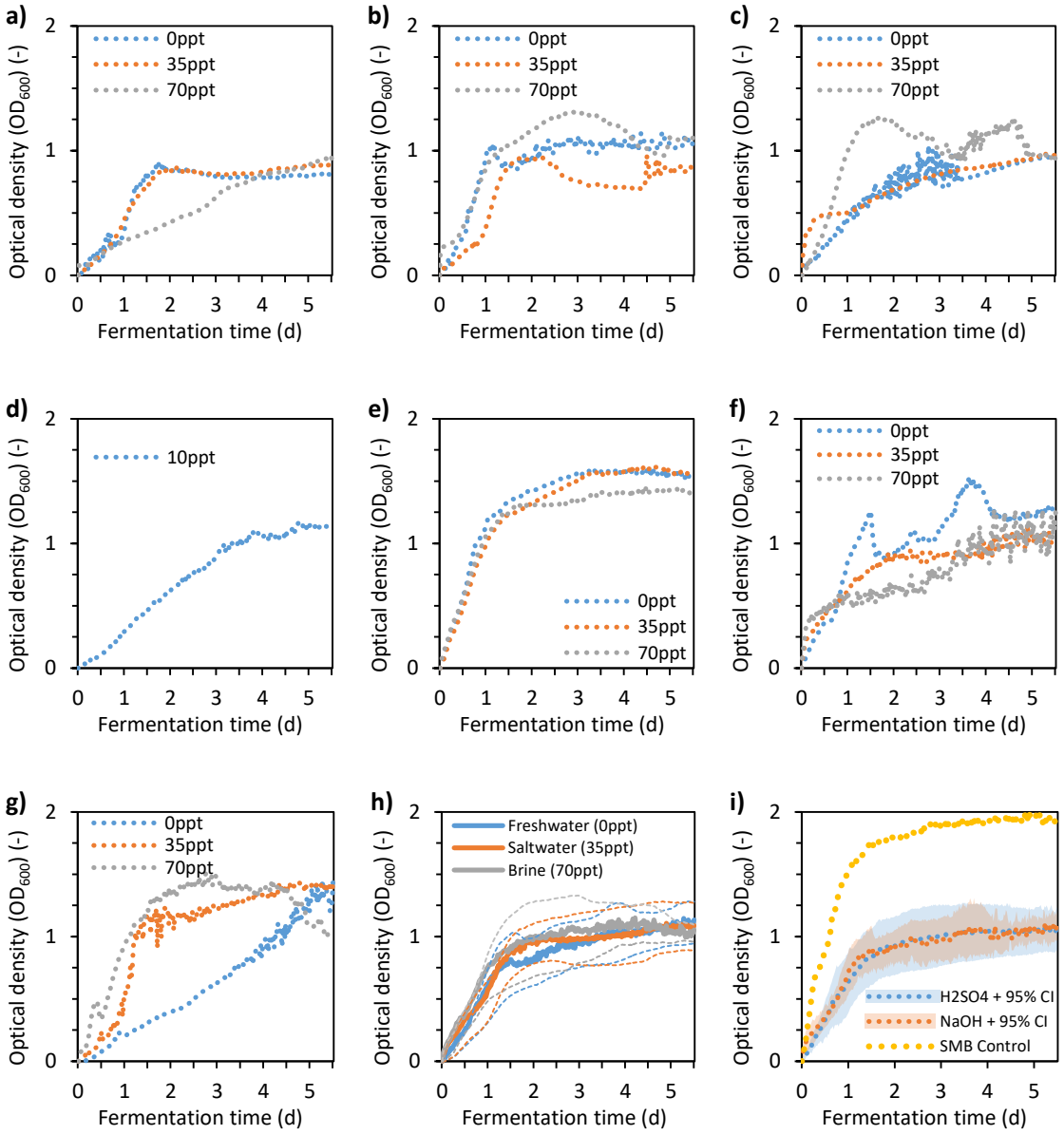

Figure 6: Average OD_{600} readings for *Metschnikowia pulcherrima* (NCYC 4331) grown on *Fucus serratus* hydrolysate produced by varied conditions in the fractionation screening experiments, for freshwater (0 ppt), saltwater (35 ppt), and heavy-brine (70 ppt): **a)** 25 °C, H_2SO_4; **b)** 25 °C, NaOH; **c)** 25 °C, no catalyst; **d)** 87.5 °C, no catalyst; **e)** 150 °C, H_2SO_4; **f)** 150 °C, NaOH; **g)** 150 °C, no catalyst; **h)** average OD_{600} by freshwater (0ppt), saltwater (35 ppt), and brine (70 ppt) sample wells, for all catalyst choices and temperatures with 95% CI about each salinity as dotted lines; and **i)** averaged OD_{600} by catalyst choice for all temperature and salinities, with shaded area representing a 95% confidence interval. OD_{600} scan

was performed once every 30 minutes, plate was incubated and agitated between reads. Colour available on-line.

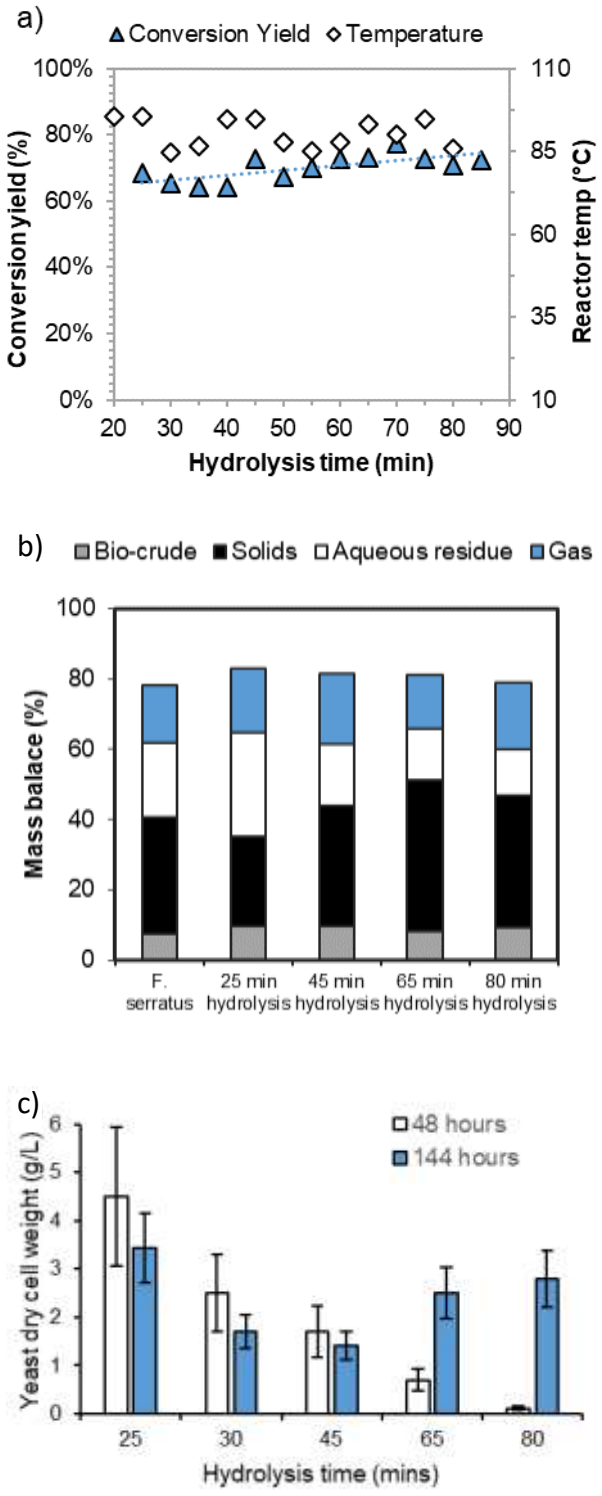

Figure 7: Conversion and subsequent valorisation of NaOH-assisted *Fucus serratus* hydrolysis on a 5 L scale, where **a)** shows the overall conversion and temperature trend over the reaction time. **b)** gives the mass balance for the resulting HTL reaction of the resulting solids after the same hydrolysis times, and **c)**

shows the dry cell weight achieved from culturing *Metschnikowia pulcherrima* on the hydrolysates produced at 48 and 144 hours. Colour available on-line.